Identification and Preliminary Characterization of Novel Small Molecules That Inhibit Growth of Human Lung Adenocarcinoma Cells

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Drug treatment for human lung cancers remains unsatisfactory, despite the identification of many potential therapeutic targets (such as mutant KRAS protein) and the approval of agents that inhibit the tyrosine kinase activity of mutant epidermal growth factor receptor (EGFR). To seek new therapeutic strategies against lung tumors, the authors have screened 189,290 small molecules for their ability to retard growth of human lung adenocarcinoma cell lines, which harbor mutations in *EGFR* or *KRAS*. Four candidates that are structurally different from common tyrosine kinase inhibitors were selected for further study. The authors describe one small molecule (designated lung cancer screen–1 [LCS-1]) in detail here. Identification of the targets of LCS-1 and other growth inhibitors found in this screen may help to develop new agents for the treatment of lung adenocarcinomas, including those driven by mutant *EGFR* and *KRAS*. (*Journal of Biomolecular Screening* 2009: 1176-1184)

Key words: high-throughput drug screen, lung cancer, EGFR, KRAS

INTRODUCTION

LUNG CANCER IS THE LEADING CAUSE OF CANCER MORTALITY IN the United States and worldwide.[1] Although surgery cures some patients when the disease is detected in its early stages, the 4 major categories of lung cancer (small-cell carcinoma, adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma) tend to metastasize readily, and none responds well to other available forms of treatment, such as radiotherapy and chemotherapy.

Efforts to subcategorize the 4 histological forms of lung cancers by genetic and biochemical methods have helped to increase our understanding of these diseases and to create opportunities for development of targeted therapies. In particular, about 10% of lung adenocarcinomas in the United States and a higher percentage in parts of Asia[2] contain mutant forms of the epidermal growth factor receptor (EGFR; usually due to a deletion mutation in exon 19 of *EGFR* or a substitution mutation, L858R, in exon 21); these changes confer sensitivity to tyrosine kinase inhibitors such as erlotinib and gefitinib.[3-5] Despite sometimes dramatic initial responses to tyrosine kinase inhibitors (TKIs), however, most tumors with EGFR mutations respond only partially, a few show little or no response, and all ultimately develop secondary drug resistance due to an additional mutation in *EGFR* (most commonly T790M),[6,7] amplification of *MET*,[8,9] or other unknown changes. Mutations in the *KRAS* gene are found in about 15% to 30% of lung adenocarcinomas,[10,11] but no successful means have been developed to target tumors with these mutations. Many other proto-oncogenes and tumor suppressor genes are now known to be mutated or show changes in copy number in lung adenocarcinomas,[12,13] but rational approaches to these putative targets are, at best, in early stages of development.

Although clues from tumor genotyping offer one attractive route to new therapeutics, high-throughput methods for assessing cancer cell phenotypes in response to small molecules or after introduction of inhibitory RNAs offer other potentially fruitful approaches. With these screening methods, it is possible to identify unsuspected vulnerabilities in cancer cells that reflect known or unrecognized mutations, changes in gene expression, or alterations in signaling pathways that create novel dependencies. For instance, mutations in genes such as *EGFR* or *KRAS* might sensitize cells to inhibition of a normally dispensable function, a phenomenon called "synthetic lethality."

We took advantage of well-characterized human lung adenocarcinoma cell lines, large libraries of small molecules, and a robotics-based screening facility to perform a survey of 189,290 small molecules for their capacity to interfere with the growth of one or more of these cell lines. In our preliminary

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screen, we used 4 adenocarcinoma cell lines with some common mutations: either of the 2 mutations in *EGFR* that sensitize cells to TKIs (the exon 19 deletion or L858R), an *EGFR* mutation that confers drug resistance (T790M) in combination with L858R, and a common mutation that activates *KRAS* (G12C). We then tested molecules confirmed to inhibit one or more of these cell lines for their ability to inhibit growth of a larger set of adenocarcinoma lines, as well as noncancerous human lung cells. Based on the phenotypic effects and their chemical properties, we selected 4 compounds for further study. We have also made a preliminary assessment of the feasibility of using 1 of the 4 selected small molecules to identify cellular targets.

MATERIALS AND METHODS

Small-Molecule Screen and Growth Assay

Chemicals were supplied by ChemBridge Research Labs (San Diego, CA; 120,000 synthetic compounds), Biofocus DPI (Saffron Walden, UK; 16,044 synthetic compounds), AnalytiCon Discovery (Potsdam, Germany; 1408 natural products), and SPECS (Delft, the Netherlands; 51,838 synthetic and natural compounds). Library compounds were preplated in 5 µL of 10% DMSO (v/v) into 384-well microplates (Corning, Corning, NY) using a liquid dispenser TPS-384 Total Pipetting Solution (Apricot Designs, Monrovia, CA); columns 13 and 14 were left empty for controls. Negative control (vehicle only) wells contained 1% DMSO (v/v), and positive control (lethal compound) wells contained 25 µM staurosporine in 1% DMSO (v/v). Cells were added in 40 µL growth medium using a bulk Multidrop Liquid Dispenser (Thermo, Waltham, MA). After a 72-h incubation, 5 µL Alamar blue reagent was added using the Multidrop Dispenser.[14] The cells were further incubated for 24 h, and the fluorescence intensity was read on either a PerkinElmer (Waltham, MA) Victor3 V multilabel plate reader (Ex: 530 nm and Em: 590 nm) or an Amersham (Fairfield, CT) LEADseeker™ Multimodality Imaging System equipped with Cy3 excitation and excitation filters and FLINT epi-mirror.[14,15] The screens against the cell lines were performed on a fully automated linear track robotic platform (CRS F3 Robot System, Thermo Electron, Gormley, Canada) using several integrated peripherals for plate handling, liquid dispensing, and fluorescence detection. Data files were loaded into the HTS Core Screening Data Management System, a custom-built suite of modules for compound registration, plating, and data management, powered by ChemAxon Cheminformatic tools (ChemAxon, Budapest, Hungary). The signal inhibition induced by the compounds was expressed as a percentage compared with high and low controls located on the same plate.

Compounds that inhibited growth by 50% or more at a concentration of 10 µM in at least 1 cell line were tested again in a confirmation screen in duplicates. The solubility and structural integrity of compounds for which growth inhibitory activity was confirmed were then tested as described under supplementary methods. Only compounds that were soluble at 100 µM were used in dose-response studies. The dose response was assessed in duplicate using 12-point doubling dilutions with a 100-µM compound concentration in 1% DMSO (v/v) as the upper limit. The dose-response curve for each set of data was fitted separately, and the 2 IC_{50} values obtained were averaged. For compounds having an IC_{50} below 1 µM, the dose-response study was repeated using dilutions starting at 10 µM for more accurate determination.

Detection of Protein Phosphorylation

For phosphorylation studies, cells were treated with compounds for 24 h in the continued presence of 10% serum. Phosphorylation was detected either by Western blotting using phospho-specific antibodies or by using the phospho-proteome profiler antibody arrays from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. For Western blotting, whole-cell extracts (50 µg) were resolved on 4% to 20% gradient gels, transferred onto nitrocellulose membranes, and then immunoblotted. For the phospho-protein antibody arrays, 300 µg of whole-cell extracts was incubated with either a mitogen-activated protein kinase (MAPK) or receptor tyrosine kinase (RTK) proteome profiler antibody array overnight.

Statistical Analysis

Assay robustness and reproducibility were estimated by the Z′ factor.[16] This statistical parameter is a function of the signal-to-noise ratio and is widely used in high-throughput screening (HTS) to validate the robustness and reproducibility of an assay. A robust cell-based assay has a Z′ factor between 0.3 and 1.0. $Z' = 3[(\sigma_{hi} + \sigma_{lo})/(\mu_{hi} - \mu_{lo})]$, where σ_{hi} and σ_{lo} are the standard deviation of the high (vehicle-treated) and low (drug-treated) controls, respectively, and μ_{hi} and μ_{lo} are the mean value of the high and low controls, respectively. Reproducibility was evaluated by performing a small screen of 3000 compounds with a wide range of biological activity on 2 separate occasions. The pair of data from the 2 validation sets was subjected to scatterplot analysis, and the standard deviation of the difference of the pairs is a measure of the assay variability. The proportion of molecules that are hits in both validation sets versus the hits in at least 1 set is the reproducibility rate. The assay was considered reproducible if the proportion of molecules that are hits in both validation sets was at least 65% of the hits observed in only 1 set.

RESULTS

Identification of Small Molecules That Reduce Growth of Human Lung Adenocarcinoma Cell Lines

We screened a library of 189,290 small molecules for agents that would inhibit growth of human lung adenocarcinoma cell

lines. The cell lines that were used in the primary screen have been well characterized and are shown in **Table 1**.[4,5,7,17,18] Three of the cell lines (H1650, H3255, and H1975) contain *EGFR* alleles found commonly in human lung adenocarcinoma cell lines (delE746-A750, L858R, and L858R-T790M, respectively). These cell lines display different sensitivity to the EGFR tyrosine kinase inhibitor gefitinib (**Table 1**). Growth of H3255 is the most sensitive to gefitinib (IC_{50} = 10 nM); this cell line also has amplification of *EGFR*.[18] In contrast, growth of H1975 (with the L858R-T790M allele) is inhibited by gefitinib only at high concentrations (IC_{50} = 10 μM). Introduction of allele-specific *EGFR* siRNAs into H1975 or H3255 induces apoptosis, suggesting that both lines require continuous expression of the *EGFR* oncogene for survival.[17] The H1650 cell line shows intermediate sensitivity to gefitinib (IC_{50} = 1 μM). The H2030 cell line contains the G12C allele of *KRAS;* growth of this line is inhibited only at high concentration of gefitinib (IC_{50} = 10 μM). Two lines of nontumor cells were used to help eliminate compounds that showed general cytotoxicity: primary human bronchiolar epithelial cells (NHBE) and human lung fibroblasts (WI-38). We sequenced exons 18-24 of *EGFR* and exon 2 of *KRAS* and did not detect any mutations in either of these 2 genes in the control cells (results not shown). NHBE and WI-38 cells showed intermediate sensitivity to gefitinib (IC_{50} = 1 μM).

Our screening strategy is outlined in **Figure 1A**. The primary screen was performed at a single drug concentration of 10 μM against H1650, H1975, H2030, and H3255. Cell growth (number of viable cells) remaining at the end of the assay was determined using the vital dye Alamar blue. This growth assay does not differentiate between cytostatic and cytotoxic compounds. Before conducting the screen, we validated the screening platform and assay by screening a library of approximately 3000 compounds against 5 cell lines (H1650, H1975, H2030, H3255, and HPL1D) on 2 separate days. The robustness and reproducibility of the assay were assessed using the Z′ statistical parameter.[16] The high signal-to-noise ratio of the assay and the Z′ values of 0.5 to 0.8 are consistent with a robust assay (see **Supplemental Table 1** at http://jbx.sagepub.com/supplemental).

Compounds that reduced growth of at least one of the adenocarcinoma cell lines at 10 μM by ≥50% were considered "positives." Using this criterion, we identified 6552 novel small molecules for further analysis (**Fig. 1B**). Out of this group, 3112 compounds were resynthesized and tested again for their ability to reduce growth, and the activity of 669 compounds was confirmed. Dose-response studies were then performed with the panel of adenocarcinoma cell lines and 2 control lines. In parallel, this group of 669 compounds was tested for solubility in growth media, and compound purity and structure were tested by liquid chromatography/mass spectrometry (LC/MS). Forty-nine compounds either had low solubility in growth media or contained impurities that raised

Table 1. Characteristics of Human Lung Adenocarcinoma Cell Lines and Control Cells

	Known Mutations		*IC_{50} for Growth Inhibition by Gefitinib, μM*
Cell Line	*EGFR*	*KRAS*	
H1650	DelE746-A750	Wild-type	1
H1975	L858R/T790M	Wild-type	10
H2030	Wild-type	G12C	10
H3255	L858R	Wild-type	0.01
WI-38	Wild-type	Wild-type	1
NHBE	Wild-type	Wild-type	1

Exons 18-24 of *EGFR* and exon 2 of *KRAS* were sequenced and the mutations identified are shown.[7] NHBE (normal human bronchiolar epithelial cells) are primary cells obtained from donor lungs and commercially available from Lonza, Inc. (Allendale, NJ). WI-38: human lung fibroblasts. The IC_{50} for inhibition of growth by the EGFR inhibitor gefitinib is shown. NHBE and WI-38 cells served as controls in the chemical screen.

questions about the identity and structure of the molecules. We were therefore able to titrate the activity of only 620 of the candidates. Fifty-nine compounds inhibited the growth of at least 1 tumor cell line with IC_{50} ≤1 μM. We then selected molecules as candidates for further study based on the following criteria: 10-fold better growth inhibition of at least 1 adenocarcinoma cell line over control cells, ease of making chemical derivatives and affinity reagents, a lack of structural similarity to adenosine triphosphate (ATP), and strong structure-activity relationships (SAR) of analogs that were present in the library. Of the 59 compounds with IC_{50} ≤1 μM for inhibition of growth of at least 1 lung cancer line, 9 showed a 10-fold selectivity (based on IC_{50} values) for tumor cells over the control cells. None of these compounds resembled ATP in structure, and there were no analogs of 5 of these 9 compounds in the library, preventing an estimate of SAR. Therefore, only 4 compounds met all of the criteria outlined above.

The growth-inhibitory profiles of these compounds are shown in **Table 2**. To simplify discussion, the 4 compounds selected for further study were given the acronym LCS (lung cancer screen)–1 to 4. All 4 impaired the growth of at least 2 adenocarcinoma cell lines with an IC_{50} <1 μM. Each of the lung cancer lines contains a known mutation affecting either EGFR or KRAS, yet the pattern of growth inhibition does not correlate with these mutations. This is most evident in the cases of LCS-2 and LCS-4. LCS-2 shows no activity against the H3255 line, but it is highly active against both the H1650 and H1975 lines, which also have *EGFR* mutations, and it is quite active against the *KRAS* mutant line, H2030. Similarly, LCS-4 is moderately active against H1975 and H2030 but shows no activity against the 2 other cell lines with *EGFR* mutations (H1650 and H3255). LCS-1 and LCS-3 inhibited growth of all 4 lines but with different patterns of activity: LCS-1 is at least 10-fold more active against H1975 than against H1650 and shows intermediate strength against H3255 and H2030; LCS-3 is most active against H1650. LCS-1 has a moderate inhibitory

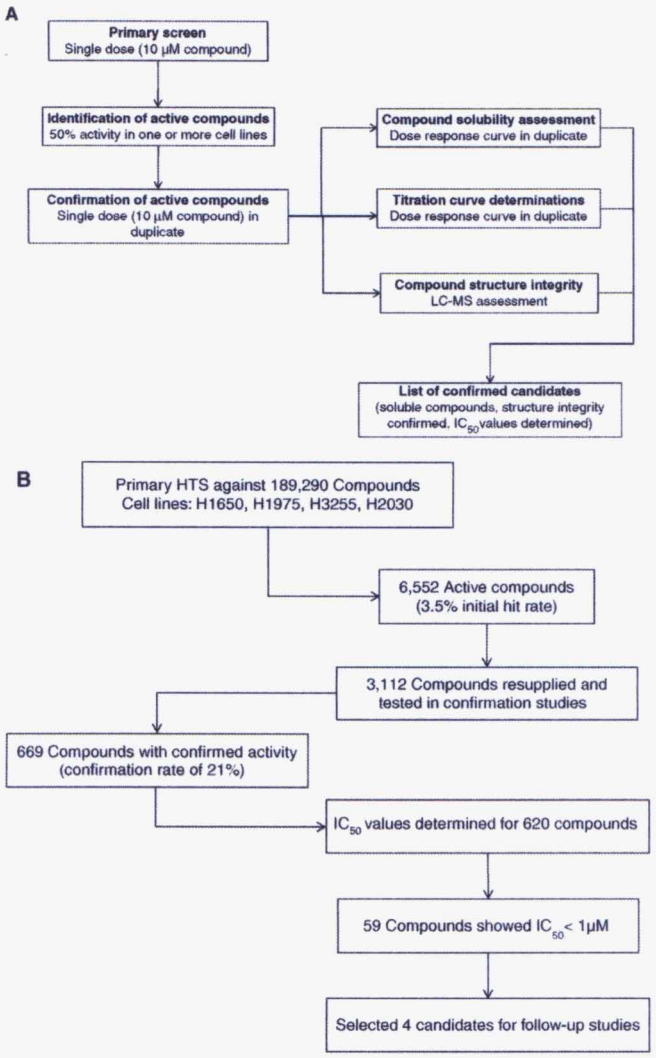

FIG. 1. Outline and summary of cell-based small-molecule screen. (A) General outline of the steps involved in screening a library of small molecules for growth inhibitors. (**B**) A library of 189,290 small molecules was screened for agents that restrict the growth of lung adenocarcinoma cell lines harboring mutations in either *EGFR* or *KRAS*. Primary screening was conducted using the 4 lung adenocarcinoma cell lines shown in **Table 1**. The dose-response studies were conducted in these lines and 2 types of control cells. Candidates for further study were selected based on the criteria described in the text.

effect on the control cell lines, but LCS-2 and -3 have at least 10-fold lower activity on NHBE cells. Because LCS-4 is inactive against 2 tumor cell lines, it was presumed not to have generalized activity against common growth mechanisms and was not tested with the control cells.

Taken together, these findings are most compatible with the idea that the active compounds are targeting the products of genes, either wild-type or mutant, on which the growth-inhibited cancer cell lines have become dependent on as a result of still unidentified mutations specific to each line. The affected proteins might or might not be components of the EGFR or KRAS signaling machinery. A satisfactory explanation of the observed patterns of sensitivity of tumor cell lines to these small molecules will probably require a fuller description of the genotypes of the cell lines and identification of the molecular targets of the compounds.

As shown in **Table 2**, the 4 candidates are all of low molecular weight and are predicted to cross the plasma membrane readily, based on the membrane partition coefficient (cLogP). The cLogP value of a compound is the logarithm of its partition coefficient between n-octanol and water ((log $C_{octanol}/C_{water}$)) and is a well-established measurement of the hydrophilicity of a compound.[19] Compounds that readily permeate the plasma membrane have a cLogP value of <5.

2-Phenylpyridazin-3(2H)-Ones as Growth Inhibitors of Human Lung Adenocarcinoma Cell Lines

The primary HTS data provided useful information about the relationship of structure to activity in the vicinity of the 2-phenylpyridazin-3(2H)-one scaffold of LCS-1. The SAR data are based on the differential activity of a cluster of 123 derivatives tested on the 4 cell lines. The SAR data strongly suggest that the pyridazine-3(2H)-one moiety of LCS-1 requires an electron-deficient environment on the pyridazine ring with closely packed substitutions such as halogens and halogen mimetics as electron-withdrawing groups tolerated at positions R_2 and R_3. Substitutions of electron-rich donors at these positions eliminated biological activity. Surprisingly, methoxy group substitutions on the R_1 position did not affect activity, whereas a hydroxyl group at the same position completely abolished activity. This observation offered the first potential point of attachment for a linker that joins an active compound to a solid support for affinity chromatography. The phenyl moiety of LCS-1 was found to tolerate the most substitutions found within the cluster identifying the R_5 position as a second potential point of attachment for a linker. The other candidates—LCS-2, LCS-3, and LCS-4—were not represented by as many analogs in the library. As shown in **Figure 2A**, LCS-1 is not related to ATP in structure and is therefore unlikely to be an ATP-competitive inhibitor. Based on its chemical structure, LCS-1 appeared to be easily amenable to chemical modification. LCS-1 was therefore the first compound chosen for further study.

Synthesis of a Focused Library of LCS-1 Derivatives

Using the SAR profile obtained from primary HTS data, we synthesized a focused library of 41 LCS-1 analogs to further explore the effects of substitutions on the R_2 and R_3 positions on the pyridazine ring and to assess the feasibility of linker attachments at the R_1 position of the pyridazine ring and at the R_5 position of the phenyl moiety of the molecule. **Supplementary Table 2** (see http://jbx.sagepub.com/supplemental) summarizes the screening data with 10 cell lines, including 2 control cell

Table 2. Cellular Activity and Chemical Properties of Candidate Small Molecules

	IC_{50} for Growth Inhibition, μM							
Compound	*H1650*	*H1975*	*H3255*	*H2030*	*NHBE*	*WI-38*	*MW*	*cLogP*
LCS-1	1.24	0.14	0.64	0.27	2.64	3.70	255	2.89
LCS-2	0.09	0.06	100	0.62	35.66	ND	231	0.83
LCS-3	0.38	0.63	0.95	1.12	21.4	ND	267	2.47
LCS-4	100	0.39	100	0.17	ND	ND	512	0.58

Cells were incubated with serial dilutions of compounds for 96 h to determine IC_{50} values for inhibition of growth. Cell growth was determined using Alamar blue vital dye. MW, molecular weight. cLogP is the partition coefficient of the molecule in a mixture of water and n-octanol, and it provides a measure of the hydrophilicity of a chemical. Compounds with low cLogP values (<5) are expected to readily cross the plasma membrane. ND, not determined.

lines. All analogs were soluble within the range of concentrations used for dose-response studies. One of the new analogs showed slightly increased potency compared to the parental molecule, 13 were inactive, and the rest inhibited growth with IC_{50} values with a potency similar to LCS-1 (see **Supplementary Table 2** at http://jbx.sagepub.com/supplemental). Chemical modifications on the R_2, R_3, R_4, and/or R_8 positions were found to tolerate only halogens or halogen mimetics, consistent with the preliminary SAR data.

The growth-inhibitory activity profile of 2 active analogs compared with the parental compound LCS-1 is shown in **Figure 2C**. Chloro-to-bromo substitutions on the R_2 and R_3 positions on the pyridazine ring resulted in the analog LCS-1.28 (4,5-dibromo-2-m-tolylpyridazin-3(2H)-one). This analog had a similar activity profile to the parental molecule. Chlorination of the ortho and the meta positions on the phenyl ring of LCS-1 produced analog LCS-1.34 (4,5-dichloro-2(2,4-dichlorophenyl)pyridazin-3(2H)-one, which was slightly more potent than LCS-1 and LCS-1.28 in inhibiting growth of H1975.

Reduction of Growth of Additional Lung Adenocarcinoma Cell Lines by LCS-1, LCS-1.28, and LCS-1.34

We expanded the panel of human lung adenocarcinoma cell lines used to test LCS-1 to include a total of 16 cell lines (8 with *EGFR* mutations and 8 with *KRAS* mutations). LCS-1 inhibited the growth of 9 of these cell lines (some with *EGFR* and some with *KRAS* mutations) at concentrations 10- to 40-fold lower than levels required to inhibit growth of NHBE or WI-38 cells (**Table 3**). When tested against 1 or 2 of the LCS-1 derivatives, the tumor cell lines were, in general, similarly sensitive to LCS-1, LCS-1.28, and LCS-1.34. However, NHBE and WI-38 cells were 2- to 3-fold more sensitive to LCS-1.28 and LCS-1.34 than to LCS-1.

Determination of the Half-Life of LCS-1, LCS-1.28, and LCS-1.34 in Human Liver Microsomes

To determine which of the three most active substituted phenyl pyridazinone compounds would be the best for in vitro and in vivo assays, we measured their stability in human liver microsomes. LCS-1 had a half-life of 6.6 min, and this was similar to the half-life of LCS-1.28 (7.1 min). LCS-1.34 exhibited a 13-fold increase in half-life (82.2 min) in human liver microsomes over the parental molecule, and it was therefore chosen for all further experiments.

Inhibition of Growth by LCS-1.34 May Be Due to Both Inhibition of DNA Synthesis and Induction of Apoptosis

To determine the cellular mechanism by which compounds related to LCS-1 reduced the number of cells, we examined the effect of the most stable analog, LCS-1.34, on DNA synthesis and apoptosis. H358, H1975, and H3255 cells were treated with increasing concentration of LCS-1.34 for 24 h, and then DNA synthesis was assessed by thymidine incorporation for the final 2 h. DNA synthesis was reduced in a concentration-dependent manner to 47% ± 3% and 42% ± 17% of control values (0.25 μM LCS-1.34) in H358 and H1975, respectively (**Fig. 3A**). H3255 was the least sensitive of the 3 cell lines, showing only a 16% reduction in thymidine incorporation (84% ± 9% of control) after treatment with 0.25 μM LCS-1.34 (**Fig. 3A**). However, at 1 μM, there was a further reduction in DNA synthesis to 30% ± 8% of control in H3255 cells, with more profound reductions in the other 2 cell lines.

To determine the effect of LCS-1.34 on cell death, we treated H358, H1975, and H3255 cells for 48 h with LCS-1.34, and annexin-FITC conjugates bound to the surface of treated cells were measured by fluorescence-activated cell sorting (FACS). LCS-1.34 induced apoptosis in the 3 cell lines to different extents (**Fig. 3B**): LCS-1.34 at 0.5 μM increased the number of H358 and H1975 cells undergoing apoptosis by 3-fold (from 8% to 24% in H358 cells and from 3.5% to 11% in H1975 cells). This concentration of LCS-1.34 did not affect the number of apoptotic cells in the H3255 cultures; however, at a higher concentration (1 μM), the number increased 2-fold, from 9% to 19%. At this higher concentration, LCS-1.34 induced an approximately 6-fold increase in the number of apoptotic cells in H358 and H1975 cultures.

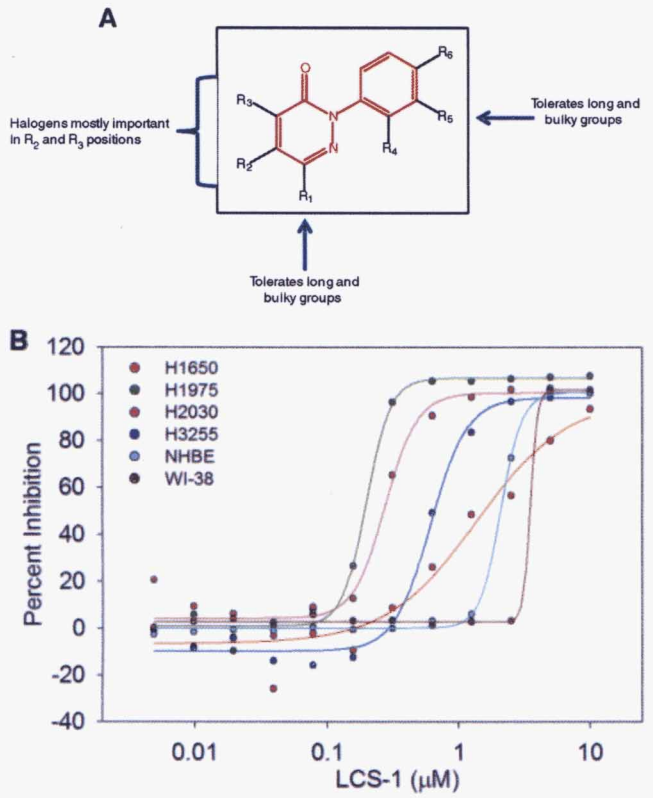

FIG. 2. Structure and growth inhibitory activity of LCS-1, LCS-1.28, and LCS-1.34. (**A**) Generalized structure of LCS-1 [4,5-dichloro-2-m-tolylpyridazin-3(2H)-one)]. A focused library of 41 LCS-1 derivatives was synthesized, and chemical modifications that were permitted are indicated. (**B**) Dose-response curves depicting the effect of LCS-1 on growth of lung adenocarcinoma cell lines and control cells. Growth was determined using Alamar blue vital dye. (**C**) The half-life of LCS-1 and 2 derivatives was determined in a preparation of human liver microsomes.

MAPK and PI 3-Kinase Pathways Are Inhibited by LCS-1.34

To gain additional insight into the mechanism by which the LCS-1-related compounds induced cell death, we analyzed the phosphorylation status of several signaling proteins that mediate proliferation and survival. We used a proteome profiler antibody array (R&D Systems) that allowed us to measure the phosphorylation status of 18 kinases, including 3 major MAPK family members (ERK, JNK, and p38) and components of the PI 3-kinase pathway (AKT, GSK-3, and p70 S6 kinase). H1975 cells were treated for 24 h with 0.5 μM LCS-1.34. Whole-cell extracts were prepared and protein phosphorylation analyzed. Phosphorylation of ERK2, the 3 AKT isoforms, and p70 S6 kinase was reduced in cells that were treated with LCS-1.34 (**Fig. 4A**). Similar results were obtained in H3255 cells (data not shown). LCS-1.34 did not, however, alter phosphorylation of any of the p38 or JNK isoforms (data not shown).

Table 3. Growth Inhibitory Profile of LCS-1, LCS-1.28, and LCS-1.34

		IC_{50} for Inhibition of Growth, μM		
Cell Line	*Mutation*	*LCS-1*	*LCS-1.28*	*LCS-1.34*
HCC827	EGFR (delE746-A750)	0.09	ND	ND
HCC4006	EGFR (delL746-E749)	0.09	ND	ND
11-18	EGFR (L858R)	0.11	0.41	ND
H1975	EGFR (L858R/T790M)	0.14	0.36	0
H358	KRAS (G13C)	0.15	0.21	0.14
H820	EGFR (delE746-E749)	0.18	0.27	0.15
H23	KRAS (G12C)	0.21	0.21	0.14
H2444	KRAS (G12V)	0.24	0.31	0.2
H2030	KRAS (G12C)	0.27	0.37	0.31
H3255	EGFR (L858R)	0.64	0.81	1.11
PC9	EGFR (delE746-A750)	0.65	0.86	ND
H2122	KRAS (G12C)	1.15	1.26	ND
H1734	KRAS (G13C)	1.17	0.8	0.52
H1650	EGFR (delE746-A750)	1.24	0.74	0.56
A549	KRAS (G12S)	1.40	1.62	ND
H460	KRAS (Q61H)	1.50	1.43	ND
NHBE	None	2.64	1.61	1.10
WI-38	None	3.70	2.61	1.74

Human lung adenocarcinoma cell lines or control cells were treated with serial dilutions of the indicated compounds for 96 h, and growth was determined using Alamar blue vital dye. ND, not determined.

EGFR Family Members Are Not Direct Targets of the Phenyl Pyridazinone Compounds

Given the effect of LCS-1 and its derivatives on some cell lines with *EGFR* mutations, we asked if phosphorylation of EGFR or other members of the EGFR family (ERBB2 and ERBB3) was affected by these compounds using a human phospho-RTK antibody array. For these experiments, we used H3255 cells, which have relatively higher levels of phospho-EGFR. Treatment of cells for 24 h with 0.5 μM LCS-1.34 did not affect total phosphorylation of EGFR, ERBB2, or ERBB3, whereas erlotinib dramatically reduced phosphorylation of all 3 receptors (**Fig. 4B**). In addition, we used a phosphorylation site-specific antiserum to show that treatment of H1975 cells with LCS-1.34 did not alter the phosphorylation of EGFR at Y1068 (**Fig. 4C**).

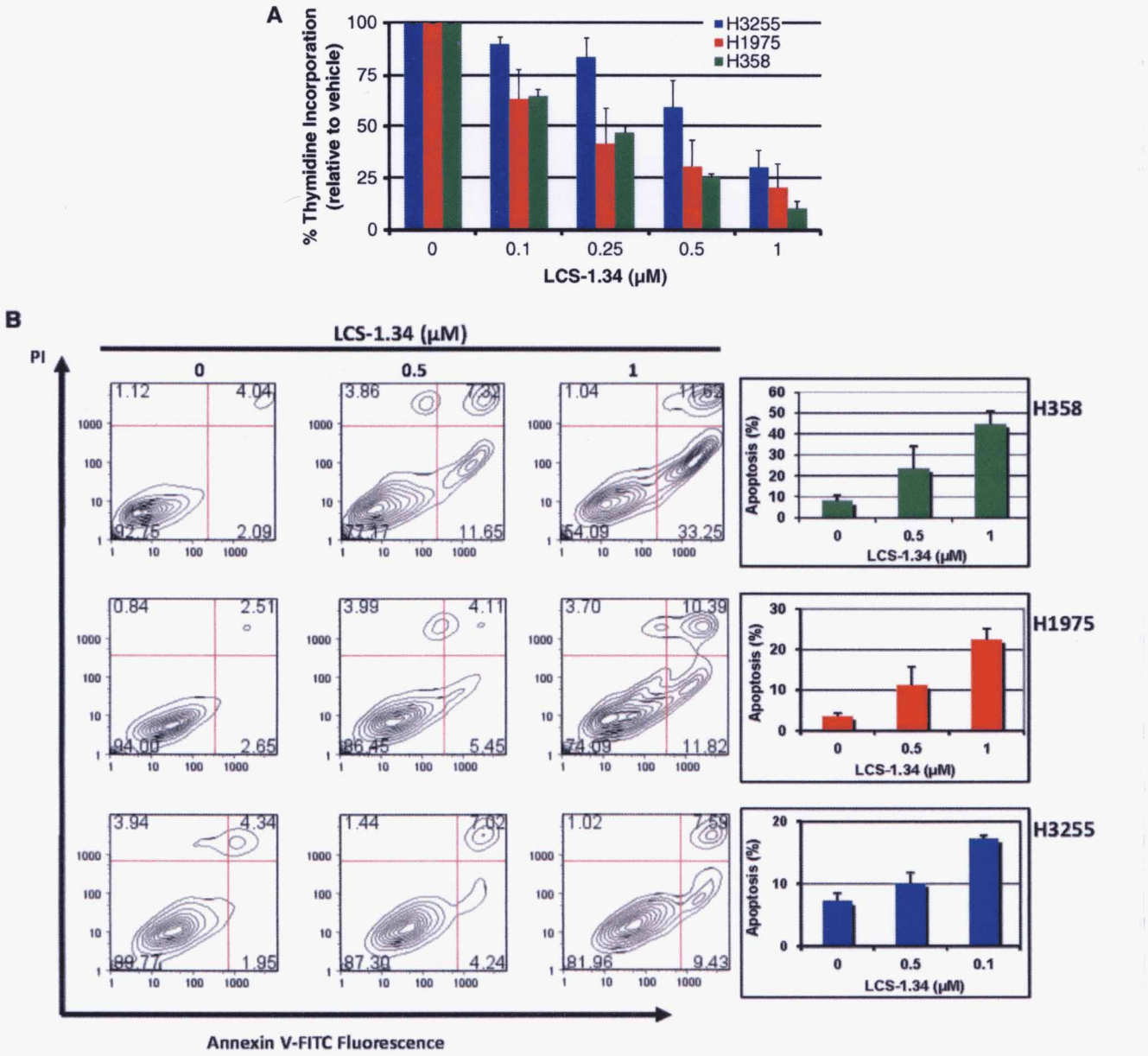

FIG. 3. LCS-1.34 inhibits DNA synthesis and induces apoptosis. Cells were treated with the indicated concentrations of LCS-1.34 for 24 h or 48 h and then thymidine incorporation or annexin V binding determined. (**A**) Results represent the mean values of 3 to 5 experiments and are expressed relative to control values (vehicle-treated cells), arbitrarily assigned a value of 100%. (**B**) Representative fluorescence-activated cell sorting (FACS) profiles are shown. The results of 4 independent experiments are shown for each cell line in the accompanying graph.

DISCUSSION

Several methods are being developed to identify new therapeutic targets in many types of cancer. These include screening with small interfering RNAs,[20] direct sequencing of genes in tumor samples,[13,21] and analysis of tyrosine phosphorylated proteins by mass spectrometry.[22,23] Here we used another approach—a cell-based, high-throughput screen of large chemical libraries—to identify novel small molecules that reduce growth of human lung adenocarcinoma cell lines and could lead to the identification of unexpected therapeutic targets.

Out of nearly 200,000 compounds, more than 6000 showed activity in our initial screen, and those were winnowed to a group of 59 compounds that had reproducible activity at sub-micromolar concentrations. We then selected 4 novel small molecules, LCS-1 to LCS-4, for further study, based on physical, chemical, and inhibitory properties, including selective activity against cancer lines as opposed to nontumor lines.

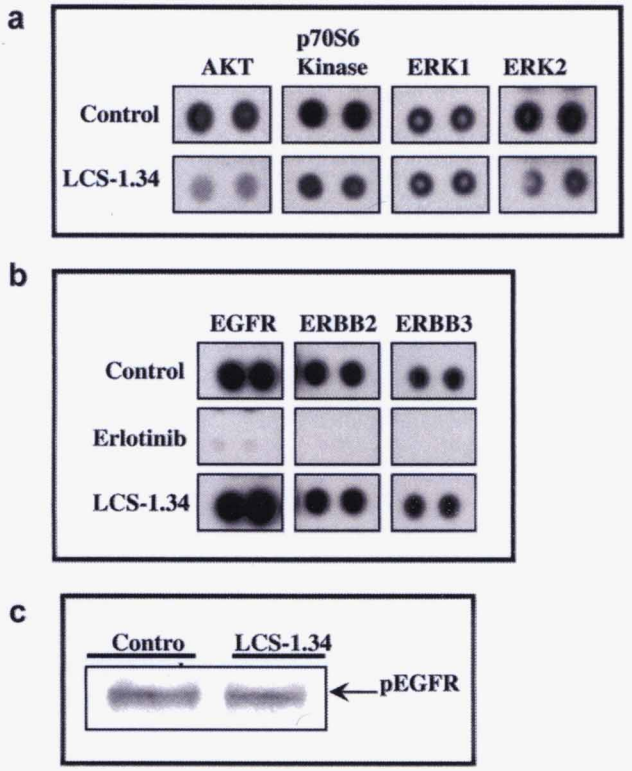

FIG. 4. LCS-1.34 blocks phosphorylation of cytoplasmic components of signaling pathways but not phosphorylation of epidermal growth factor receptor (EGFR) family members. H1975 or H3255 cells were treated with 0.5 μM LCS-1.34 for 24 h, and then cell extracts were prepared. Each experiment was repeated at least twice. (**A**) The phosphorylation status of cytoplasmic kinases in H1975 cell extracts was determined using a human phospho-MAPK proteome profiler antibody array. Each phospho-protein is represented in duplicate. (**B**) Phosphorylation of the EGFR family members in H3255 was determined using a receptor tyrosine kinase phospho-protein profiler array. (**C**) Phosphorylation of EGFR in H1975 was analyzed by Western blot using a phospho-specific EGFR (Y1068) antibody. A representative blot is shown.

Our efforts to exploit the results of the screen were focused on characterizing LCS-1. This 2-phenylpyridazin-3(2H)-one-based compound was amenable to chemical modification, allowing us to make a derivative (LCS-1.34) that was 13-fold more stable in human liver microsomes. We tested LCS-1, LCS-1.34, and one other derivative for growth-inhibitory activity against a panel of 16 human lung adenocarcinoma lines with *EGFR* or *KRAS* mutations and observed antitumor cell activity in over half of the lines. However, there was no correlation of the growth-restricting activity with known mutations in *EGFR* or *KRAS*. Until more is known about the genetic and biochemical features of these cell lines, we will be unable to make any definitive correlation between genotypes or signaling activity and sensitivity to LCS-1 and its derivatives.

Reduced cell growth was associated with partial inhibition of DNA synthesis and with partial induction of apoptosis in the lines tested, but the major physiological actions of LCS-1 or other agents have not been established. In addition, we do not know whether inhibition of one or more of the well-characterized signaling pathways is responsible for the effects observed on DNA synthesis and apoptosis. In some initial surveys of biochemical activities, LCS-1 impaired phosphorylation of MAPK, AKT isoforms, and p70 S6 kinase, enzymes that act in pathways known to regulate cell growth and survival. But until we have undertaken a more extensive investigation of the effects of our 4 compounds, we cannot draw any conclusions about whether the effects on phosphorylation reported here are responsible for the effects on cell growth that allowed us to select these compounds. Notably, LCS-1 does not appear to affect the activation of EGFR family members, as measured by phosphorylation status; consequently, if inhibition of the MAPK and PI 3-kinase reflects interference with the EGFR signaling pathway, the effects likely occur at some step downstream of the receptor. Alternatively, these effects could occur in a manner independent of the EGFR pathway.

Finally, we propose that the compounds we have identified here as tumor cell-specific inhibitors of growth could lead to identification of their cellular targets by affinity chromatography[24] or by methods based on perturbation of gene expression profiles.[25] Knowledge of such targets could deepen our understanding of how oncogenic EGFR and KRAS transform lung epithelial cells, provide specific targets for cancer drug development, and predict how cells might escape the growth-inhibitory effects of the candidate small molecules described here. Indeed, small molecules have been used to identify targets of activated KRAS in smaller screens.[26] Because the small molecules that we selected for further study do not resemble ATP structurally, they may not be ATP-competitive inhibitors. However, this will have to be tested experimentally. It is possible that the targets of our compounds are nontraditional drug targets, such as nonenzymatic components of cytoplasmic signaling networks or transcription factors.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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